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# Induction of avirulence by AVR-Pita1 in virulent U.S. field isolates of *Magnaporthe oryzae*

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## ABSTRACT

The AVR-Pita1 gene, from the Chinese isolate O-137 of *Magnaporthe oryzae*, is an effector that determines the efficacy of the Pi-ta rice blast resistance gene. In the present study, the avirulence function of AVR-Pita1 was induced by transformation of field isolates (TM2, ZN19, B2 and B8) that originally were collected from the U.S. and are virulent on Pi-ta-carrying rice cultivars. The presence of AVR-Pita1 from O-137 in independent transformants was detected by PCR using AVR-Pita1 specific primers and verified by DNA sequencing and Southern blot analysis using the AVR-Pita1 coding region as a probe. The results of pathogenicity assays showed that the AVR-Pita1-transformed isolates were not able to infect rice cultivars Katy and Drew carrying Pi-ta. Control isolates that were transformed with inserts lacking the AVR-Pita1 gene remained virulent. Our findings demonstrate that AVR-Pita1 can be used to induce novel gene-specific blast resistance in nature.

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## 1. Introduction

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (formerly *Magnaporthe grisea*), is one of the most destructive diseases of rice worldwide. The fungus can also cause severe infections in wheat [1]. Avirulence (AVR) genes in *M. oryzae* are known to be highly unstable [2] owing to the presence of a large number of active transposable elements that can shape the evolution and adaptation of the fungus [3]. To date, seven race-specific avirulence genes, AVR-Pita1, AVR1-CO39, ACE1, AVR-Pizt, AVR-Pik, AVR-Pii and AVR-Pia, have been

molecularly characterized. ACE1 has been found among rice isolates and can be used to induce avirulence [4]. It encodes a putative polyketide synthase fused to a non-ribosomal peptide synthetase determining the specificity of Pi33 [4]. AVR1-CO39, an isolate of *M. grisea* from weeping lovegrass, encodes a signal that triggers a strong defense response in the rice cultivar CO39, which carries the corresponding resistance (R) gene [5]. AVR-Pizt, recognized by the R gene Piz-t, can suppress BAX-mediated programmed cell death in *Nicotiana benthamiana*, suggesting a mechanism by which AVR-Pizt may confer virulence on *M. oryzae* [6]. The remaining three AVR genes of *M. oryzae* were novel genes

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[7]. In contrast, abundant major and minor blast resistance (R) genes have been identified in rice germplasm worldwide [8]. Thus far 16 major and two minor blast R genes have been cloned, most of which encode NBS type R proteins [9].

Understanding R-AVR gene interaction specificity is important for the development of effective strategies to manage rice blast disease. AVR-Pita determines the efficacy of the R gene Pi-ta [10]. The genes Pi-ta and AVR-Pita are the first R-AVR gene pair characterized in the rice blast system. AVR-Pita is located in the telomeric region of chromosome three of *M. oryzae*, and was cloned from a Chinese isolate, O-137 [10]. AVR-Pita was renamed AVR-Pita1 following the discovery that it has several family members in the *Magnaporthe* species [11]. AVR-Pita1 encodes a protein with 223 amino acids with properties highly similar to those of a metalloprotease [10]. AVR-Pita716 codes for a putative product that was predicted to be an elicitor that binds to Pi-ta directly inside plant cells, triggering an effective blast resistance response [12,13]. Other biological functions of AVR-Pita in *M. oryzae* remain unclear; however, it was predicted to be involved in the early stages of pathogenesis [10]. Recently, AVR-Pita1 was shown to accumulate in the biotrophic interfacial complex (BIC), raising the possibility that AVR-Pita1 prepares rice cells for subsequent fungal entry and biotrophic growth [14]. A recent analysis of strains of *M. oryzae* worldwide revealed that the organization of AVR-Pita1 and its family members is complex in the *M. oryzae* species [15].

Pi-ta encodes a NBS-LRD receptor protein with a single amino acid, alanine, at position 918 of the Pi-ta protein that determines resistance specificity [12]. In the U.S., Pi-ta from the Vietnamese cultivar Tetep has been successfully introgressed into a series of high-yielding and good-quality rice cultivars including Katy, Drew, Madison, Kaybonnet, Cybonnet, Ahrent, Banks, and Spring using classical plant breeding in conjunction with marker-assisted selection since the 1990s [16–25]. To date a total of 11 rice cultivars with Pi-ta have been released in the southern U.S. [9].

Studying the structural and functional integrity of AVR-Pita1 genes in *M. oryzae* is important in order to predict the stability of the deployed Pi-ta gene. For example, the Pi-ta gene deployed in the high yielding cultivar Banks (and other previously mentioned cultivars) was defeated by a virulent race, IE-1k, resulting in serious crop losses in Arkansas [26]. The fungal isolates from this field were determined to contain altered AVR-Pita1 alleles [27]. Additionally, it was demonstrated that avirulent isolates on Pi-ta could become virulent on the same cultivars after a single round of inoculation and selection [28]. It is unknown whether AVR-Pita1 from O-137 can induce avirulence in virulent isolates found in commercial rice fields in the southern U.S.

The objectives of the present study were to 1) create transformants with AVR-Pita1 using PEG-mediated recombination; 2) verify the presence of AVR-Pita1 using PCR, DNA sequencing, and Southern blot analysis; 3) evaluate disease reactions on rice cultivars with and without Pi-ta, and 4) identify the functional domains of AVR-Pita1 protein variants in the U.S. field isolates. The ultimate goal of this study is to develop a deeper understanding of plant-pathogen interaction that may lead to novel resistance mechanisms that can be genetically engineered in plants.

## 2. Materials and methods

### 2.1. Rice materials and growth

The cultivars Katy and Drew, carrying Pi-ta, and the cultivars M202, Francis, and Wells, lacking Pi-ta, were used for the present study [16,18,23–25,29]. Plants were grown in the growth chamber at a temperature range of 24 to 32 °C under a photoperiod of 16 h/8 h (light/dark). Cultivars at the 4-leaf stage were used for inoculation.

### 2.2. Fungal transformation

Plasmid PCB980, carrying a 490 bp promoter plus the open reading frame of AVR-Pita, and plasmid PCB1003, carrying hygromycin B (HyB) resistance, [10] were co-introduced into protoplasts by PEG-mediated transformation with the following modifications: mycelia were grown on an oatmeal plate for two weeks and then 2.5 cm square blocks were sliced and blended for 30 s in 50 mL YEG (20 g glucose and 5 g yeast extract per 1 L distilled water) containing 50 µg mL<sup>-1</sup> ampicillin for protoplast formation. The mixture was then transferred to a 250 mL flask and shaken overnight at room temperature. The culture was then blended for 30 s and poured back into the same flask containing 50 mL complete medium with 50 µg mL<sup>-1</sup> ampicillin. The inoculated flask was shaken overnight at room temperature to produce protoplasts. Protoplasts were collected by filtering through a layer of sterilized Miracloth, washed with 1 mol L<sup>-1</sup> sorbitol twice and then resuspended in 50 mL 1 mol L<sup>-1</sup> sorbitol containing 1 mg mL<sup>-1</sup> NOVOZYM lysing enzyme (Sigma-Aldrich, St. Louis, MO), and incubated at 30–32 °C for 1.5 h with shaking at 60 r min<sup>-1</sup>. Protoplasts were recovered from the Miracloth by filtering through one layer of Miracloth and rinsed with 50 mL of 1 mol L<sup>-1</sup> sorbitol. Finally, protoplasts were washed twice with 1× STC (20% sucrose, 50 mmol L<sup>-1</sup> Tris-HCl, pH 8.0, and 50 mmol L<sup>-1</sup> CaCl<sub>2</sub>) by centrifugation at 4500 r min<sup>-1</sup> for 6 min and the final concentration was adjusted to 5 × 10<sup>7</sup> protoplasts mL<sup>-1</sup>. As a control, four isolates were transformed with the selectable marker (PCB1003) alone using the previously described protocol to determine whether the transformation and protoplast process had any effect on virulence.

PCB980 (4 µg in 25 µL H<sub>2</sub>O) and PCB1003 (1 µg in 25 µL H<sub>2</sub>O) were mixed with 200 µL protoplast solution in a 15 mL Falcon tube and incubated at room temperature for 20 min. Then 1 mL of PTC (40% PEG8000 in 1× STC, prepared fresh and filter-sterilized) was added to the tube, mixed by inverting the tubes several times and then incubated at room temperature for 20 min. Next, 5 mL TB3 (3 g yeast extract, 3 g casamino acids, and 20% sucrose per 1 L of H<sub>2</sub>O) was added with 50 µg mL<sup>-1</sup> of ampicillin, shaken overnight at room temperature at 80 r min<sup>-1</sup>, and spun down at 5000 r min<sup>-1</sup> for 5 min. The solution was resuspended in 200 µL STC and divided into two tubes: 20 µL in one and 180 µL in the other, for transformation. Ten milliliter containing 0.7% (W/V) low-melting temperature agarose was melted in TB3 by a microwave oven, and cooled to 47–55 °C. Ampicillin (final concentration: 50 µg mL<sup>-1</sup>) and HyB (final concentration: 250 µg mL<sup>-1</sup>) were added to low-melting agarose for two Petri dishes. The Petri dishes were incubated at room temperature overnight, overlaid with 10 mL low-melting

agarose, and incubated at room temperature for 4 days. Surviving mycelia were identified, transferred to an oatmeal agar Petri dish containing  $150 \mu\text{g mL}^{-1}$  of HyB, and purified.

### 2.3. DNA extraction, primers, PCR amplification, and sequencing

Mycelia were grown in a liquid complete medium (6 g of yeast extract, 6 g of casein acid hydrolysate, and 10 g of sucrose per 1 L of distilled water) for 7 days. Mycelia were collected, dried under vacuum overnight, and stored at  $-80^\circ\text{C}$ . DNA of *M. oryzae* was isolated from dried mycelia using the CTAB method [29]. One forward and one reverse primer from the 5' and 3' ends of the AVR-Pita1 gene, YT4 (5'-ACATCGATGCTT TTTTATTC-3') and YT5 (5'-ACGGATCCTTAACAATATTTATAA CGTGAC-3'), were used to amplify the coding region of AVR-Pita1 from PCB980 [10] for use as a probe for Southern blot analyses. All PCR reactions were performed using Taq PCR Master Mix (Qiagen Inc., Valencia, CA, USA). Each PCR reaction consisted of the following components: 6.25  $\mu\text{L}$  of Taq PCR Master Mix, 0.25  $\mu\text{L}$  of each  $10 \mu\text{mol L}^{-1}$  primer, 10 ng of fungal genomic DNA, and distilled water (provided by Qiagen Kit) in 12.5  $\mu\text{L}$ . Reactions were performed in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA, USA) with the following PCR program: 1 cycle at  $95^\circ\text{C}$  for 3 min for initial denaturation, 35 cycles at  $95^\circ\text{C}$  for 30 s,  $55^\circ\text{C}$  (varying with different primer pairs) for 30 s,  $72^\circ\text{C}$  for 30 s, and a final extension of  $72^\circ\text{C}$  for 7 min. The PCR products were separated by electrophoresis on 1.0% (W/V) agarose gels in  $1\times$  TAE buffer mixed with 10% (V/V) SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA), and visualized and photographed with a Bio-Rad Gel Photographic System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All PCR reactions were repeated twice.

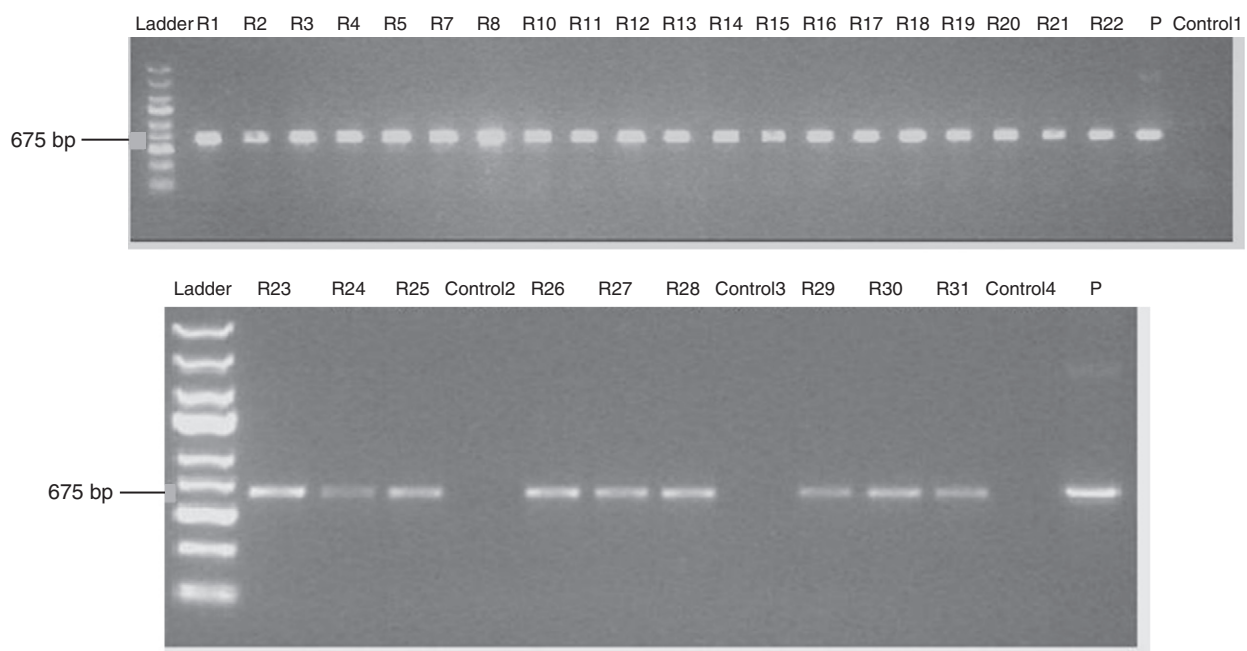
PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions for sequencing. DNA sequencing was performed with the same primers for PCR amplification, YT4 and YT5, in an ABI 3730XL DNA Analyzer using 0.5  $\mu\text{L}$  BigDye v3.1 at the USDA-ARS MSA Genomics Laboratory at Stoneville, MS.

### 2.4. Southern hybridization

The restriction endonuclease *Bam*H I was used for digestion of genomic DNA. Digested genomic DNA was electrophoresed on 0.8% agarose gels in  $1\times$  TAE buffer and transferred onto Hybond-N<sup>+</sup> nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ, USA) following the protocols described by [30,31]. The probe for the AVR-Pita1 coding region was produced by amplifying the coding region of AVR-Pita1 from Plasmid PCB980 using YT4 and YT5. DIG-labeled DNA probes were generated using a PCR DIG Probe Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA), hybridized to blots overnight at  $41^\circ\text{C}$ , and washed under high-stringency conditions according to the manufacturer's instructions. The detection and stripping methods followed the instructions in the manual "DIG-High Prime DNA Labeling and Detection Starter Kit I" from Roche Applied Science. The size of the fragment was measured against the DNA Molecular Weight Marker II, Digoxigenin-labeled (Roche Diagnostics Corporation, Indianapolis, IN, USA).

### 2.5. Fungal preparation and pathogenicity assays

In contrast to IA45, IB1, IB45, IB49, IC17, ID1, IG1, IE1 and IH1, isolates TM2, ZN19, B2 and B8 were virulent on *Pi-ta*-carrying cultivars [26,31]. These four isolates were stored at  $-20^\circ\text{C}$  on



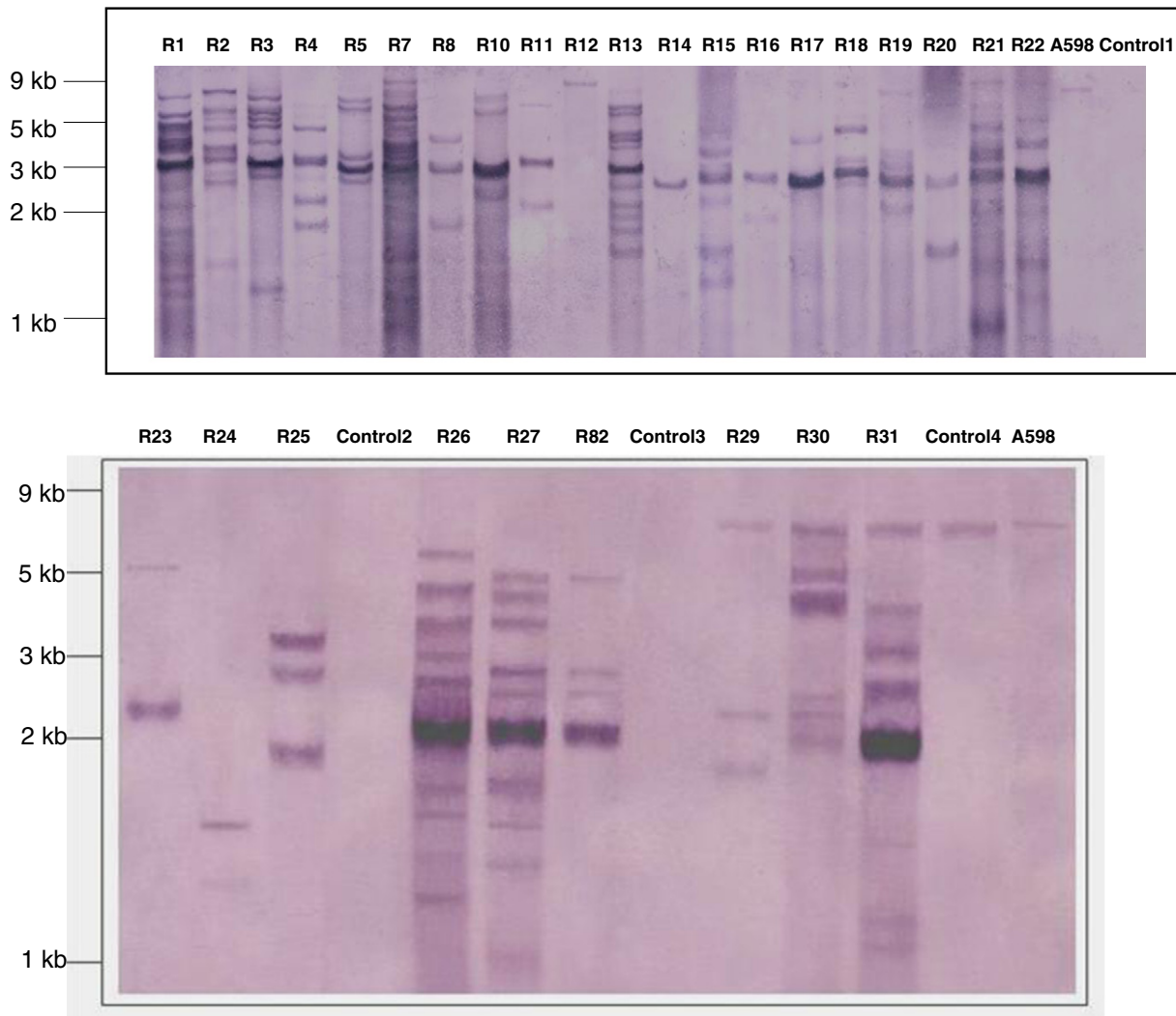
**Fig. 1 – PCR amplification of AVR-Pita1 in transformants.** The names of transformants R1 to R31 except for R6 and R9 are indicated. Plasmid PCB980 (indicated as P) and non-AVR-Pita1-carrying transformants were used as positive and negative controls (indicated as controls 1–4) respectively. The size of amplified fragment was estimated with a GeneRulerExpress DNA Ladder (Fermentas Inc. Glen Burnie, MD, USA).

desiccated filter paper, and were grown either at room temperature under blue and white fluorescence lights on plates containing oatmeal agar for production of conidia, or in complete medium broth at 24 °C for producing mycelia for DNA preparation [27]. Twenty-nine PCB980 introduced transformants and non-PCB980-containing transformants were evaluated on Katy, Drew and M202. Pots of the three cultivars were grown in a completely randomized design and seedlings were inoculated with and without PCB980 transformants. Conidial concentration was measured with a hemacytometer and adjusted to  $2 \times 10^6$  conidia  $\text{mL}^{-1}$  for inoculation. Five seedlings per pot (6 cm diameter) were inoculated at the 4-leaf stage with 15 mL of conidial suspension ( $2 \times 10^6$  conidia  $\text{mL}^{-1}$ ) using an airbrush sprayer. After inoculation, the seedlings were placed in sealed plastic bags at room temperature to maintain 70%–90% humidity. Rice seedlings were returned to the greenhouse 24 h after incubation. Disease reactions were recorded 7 days post-inoculation using a 0–5-scale rating system (0–1: resistance; 2–5: susceptible) [32]. Each experiment was repeated three times.

### 3. Results

#### 3.1. Isolation of AVR-Pita1 transformants using PEG-mediated transformation

AVR-Pita1, in the plasmid vector PCB980, was co-introduced with plasmid PCB1003, containing the selection marker HyB resistance, using PEG-mediated transformation. AVR-Pita1 was introduced into four isolates, TM2, ZN19, B2 and B8. A total of 100 putative recombinant fungal colonies were grown on HyB-containing media. The 100 colonies were isolated and stored for subsequent experiments. As a control, four isolates were transformed with the selectable marker (PCB1003) alone to determine whether the transformation and protoplast process had any effect on virulence. To confirm that AVR-Pita1 had been introduced into the protoplasts regenerated from isolates TM2, ZN19, B2 and B8, PCR using the primer pair YT4/YT5 was used to amplify the AVR-Pita1 coding region from 100



**Fig. 2 – Identification of AVR-Pita1 in transformants using Southern blotting.** Genomic DNA of indicated AVR-Pita1-carrying transformants, the isolate A598 (avirulent isolate) and non-AVR-Pita1-carrying transformants was digested with *Bam*H I and blotted with Hybond-N<sup>+</sup>. The coding region of AVR-Pita1 was used as probe using a DIG-labeling method.



putative transformants. Plasmid PCB980 containing AVR-Pita1 was used as a positive control and genomic DNA from the non-AVR-Pita1-containing transformants (without PCB980) was used as a negative control. A total of 29 transformants were identified by PCR screening as carrying newly introduced AVR-Pita1 (Fig. 1). A PCR product of 675 bp was repeatedly amplified in both the 29 putative transformants and the positive control (Fig. 1). No product was amplified from the negative control. These results indicate that AVR-Pita1 was successfully introduced into the virulent U.S. isolates.

To verify the identity of AVR-Pita1 in the transformants, amplified PCR products were sequenced and verified with the sequence of AVR-Pita1 amplified from PCB980. Both sequences of AVR-Pita1 were identical, suggesting that the PCR product amplified was from the AVR-Pita1 coding region originally cloned in PCB980.

To determine the copy number of transformants, the AVR-Pita1 coding region was used as a probe for Southern blot analysis. Multiple hybridization bands of different sizes were found in most transformants. These results suggested that the copy numbers of transformants varied from one copy

in recombinant R12 to 15 copies in recombinant R1 (Fig. 2). Partial incorporation of the transgene occurred in some cases, given that some bands with size around 1 kb (<1.5 kb AVR-Pita1 promoter plus coding region) appeared on the membrane (Fig. 2).

### 3.2. Pathogenicity assays of transformants

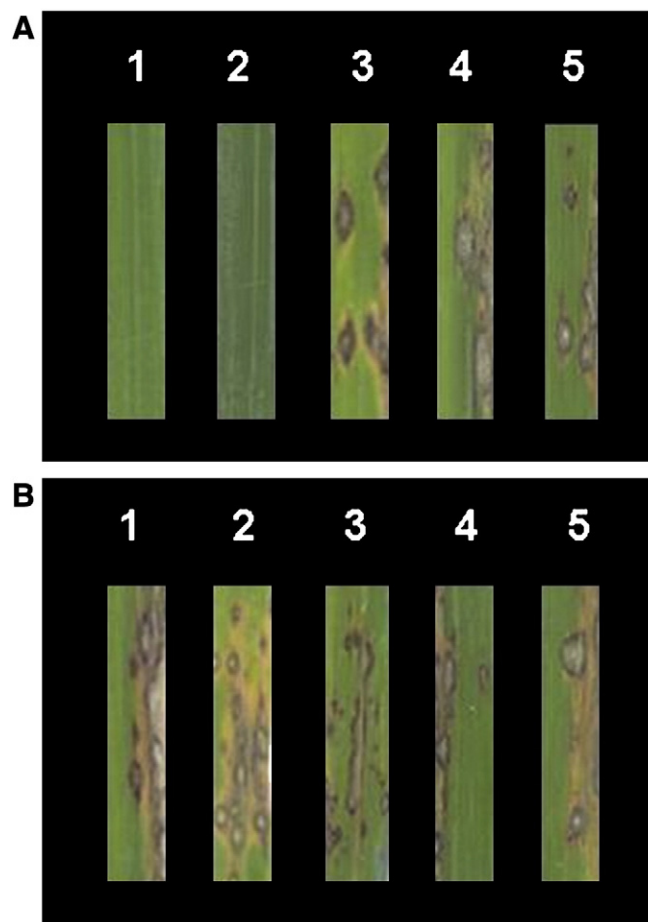
A standard pathogenicity assay [2] was applied using Pi-ta-carrying rice cultivars Katy and Drew and non-Pi-ta-containing rice cultivars M202, Wells and Francis (Table 1). Typical blast disease symptoms were observed on M202, Wells, and Francis, and were not observed on Katy and Drew when transformants were used for inoculation (Fig. 3). As a control, blast disease was observed on all cultivars when non-PCB980-carrying transformants were used for inoculation. These results demonstrated that all the PCB980-introduced transformants became avirulent toward the Pi-ta-containing cultivars Katy and Drew but not toward the non-Pi-ta-containing cultivars M202, Wells, and Francis (Fig. 3). Each test was repeated three times with the same results.

**Table 1 – Disease reactions of cultivars with and without Pi-ta to recombinant isolates and controls.<sup>a</sup>**

Transformant	Original isolate regenerated from <sup>b</sup>	Cultivar				
		Katy (+Pi-ta)	Drew (+Pi-ta)	M202 (–Pi-ta)	Wells (–Pi-ta)	Francis (–Pi-ta)
R1	TM2	0	0	5	5	5
R2	TM2	0	0	5	4	5
R3	TM2	0	0	5	5	5
R4	TM2	0	0	5	5	5
R5	TM2	0	0	5	4	5
R7	TM2	0	0	5	5	5
R8	TM2	0	0	5	5	4
R10	TM2	0	0	5	5	5
R11	TM2	0	0	5	5	5
R12	TM2	0	0	5	5	5
R13	TM2	0	0	5	5	5
R14	TM2	0	0	4	5	4
R15	TM2	0	0	5	5	5
R16	TM2	0	0	4	4	5
R17	TM2	0	0	5	5	5
R18	TM2	0	0	5	5	4
R19	TM2	0	0	5	4	4
R20	TM2	0	0	5	5	5
R21	TM2	0	0	5	5	5
R22	TM2	0	0	5	5	5
R23	ZN19	0	0	5	5	5
R24	ZN19	0	0	4	5	5
R25	ZN19	0	0	5	5	5
R26	B8	0	0	5	5	5
R27	B8	0	0	5	5	5
R28	B8	0	0	5	4	5
R29	B2	0	0	5	5	5
R30	B2	0	0	4	5	5
R31	B2	0	0	5	5	5
Control 1	TM2	5	5	5	5	5
Control 2	ZN19	4	5	5	5	5
Control 3	B8	5	5	5	5	5
Control 4	B2	5	4	5	5	5

<sup>a</sup> Controls 1–4 were fungal isolates regenerated using the same procedure with hygromycin B marker and without AVR-Pita1. 0–1: resistant; 2–5: susceptible [2].

<sup>b</sup> Original isolate sources from which the transformants were regenerated.



**Fig. 3 – Disease reactions to *M. oryzae* transformants of rice cultivars with and without *Pi-ta*. A. Inoculation of AVR-Pita1-containing recombinant onto the cultivars Katy (1), Drew (2), M202 (3), Wells (4) and Francis (5), respectively. B. The same set of cultivars was inoculated with a non-AVR-Pita1-carrying recombinant.**

### 3.3. Sequence analysis of AVR-Pita1 variants

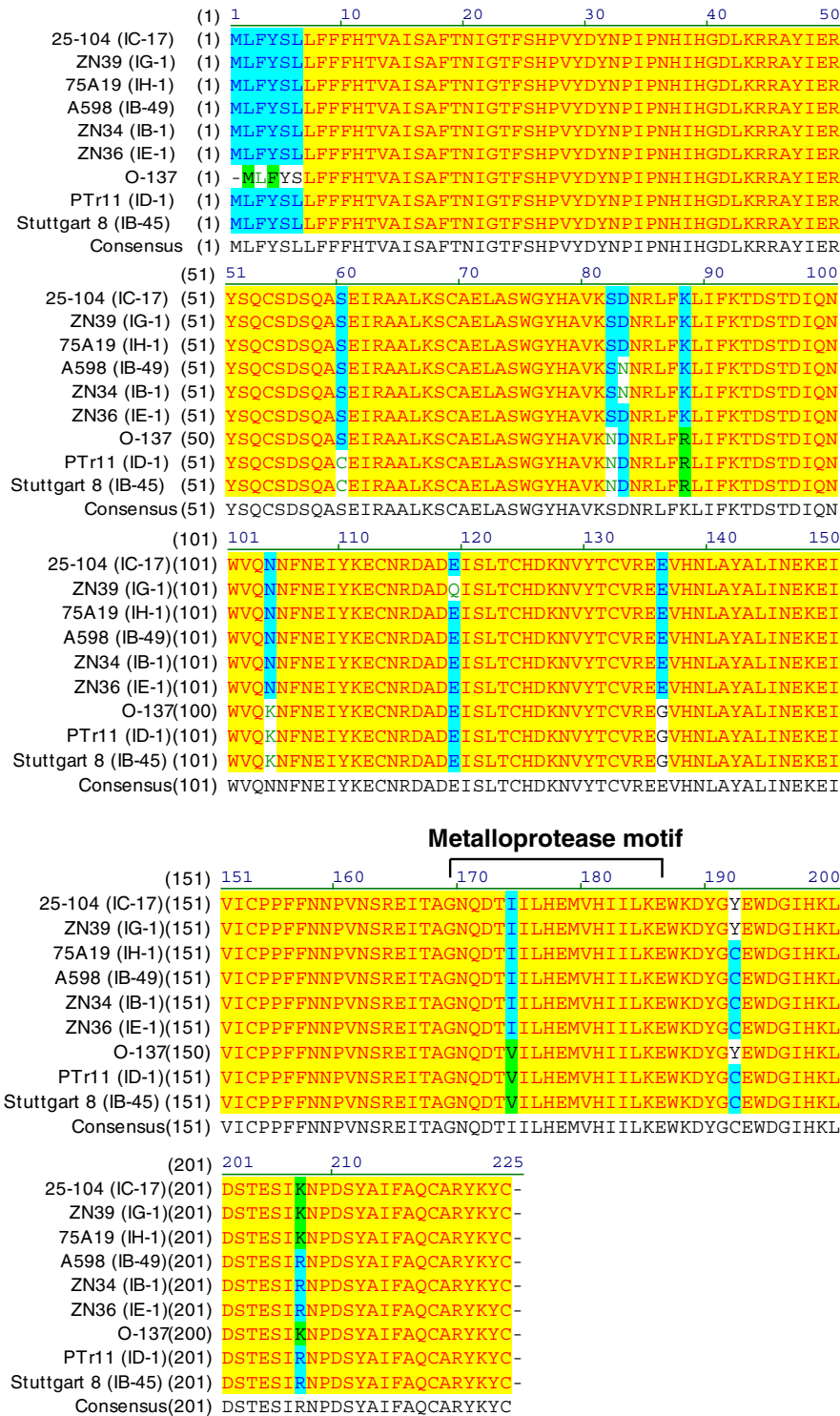
*Pi-ta* was previously known to confer resistance to races IA45, IB1, IB45, IB49, IC17, ID1, IG1, IE1 and IH1 [32]. To identify important domains among AVR-Pita1 variants in these races, amino acid sequences were aligned using Vector NTI software (Invitrogen, Eugene, OR, USA). Alignments of all amino acid sequence assemblies revealed 92.4% identity. The differences were at positions 5, 59, 81, 82, 87, 103, 119, 135, 173, 191 and 206 (Fig. 4). It is important to note that the substitution V173I lies in a zinc metalloprotease motif with little protein-structure change, given that both valine and isoleucine are hydrophobic. Since all isolates described in Fig. 4 were avirulent to rice germplasm carrying *Pi-ta*, the amino acid variation in the isolates has no apparent influence on the avirulence activity of AVR-Pita1.

## 4. Discussion

Continuing challenges in crop protection lie ahead, owing to the rapid appearance of more virulent strains of various pathogens. This is particularly true for the rice blast pathogen. Although rice cultivars containing the broad-spectrum *Pi-ta* gene have

been developed and effectively deployed, occasionally blast disease still results in serious crop losses under favorable conditions in the southern U.S. For example, the high-yielding cultivar Banks, which carries the *Pi-ta* gene, was severely infected by *M. oryzae* in Arkansas in 2004 [26]. Subsequently, seven virulent isolates, B2 to B8 of *M. oryzae*, were identified in this rice field. Not surprisingly, the deletion of the AVR-Pita1 gene in these seven isolates was able to avert recognition and detection by the *Pi-ta* gene [27].

In the past, pathologists have relied on field isolates of the common U.S. races IC17, IB49, IG1, IH1, IB1, IE1 and ID1 to evaluate the *Pi-ta* resistance spectrum [32]. Isolates overcoming resistance in *Pi-ta* carrying rice cultivars were predicted to lack avirulence toward *Pi-ta*. PCR analysis using AVR-Pita1-specific alleles and Southern blot analysis using portions of AVR-Pita1 as probes suggest that the function of AVR-Pita1 was lost in virulent isolates [27]. In the present study, two of these virulent isolates (B2 and B8) were used as recipients along with TM2 and ZN19 (race IE1k), and 29 transformants were generated that contained AVR-Pita1 from a Chinese isolate O-137, from which AVR-Pita1 was originally isolated [10]. The transformants became avirulent on rice cultivars that contained *Pi-ta*. For the first time we have demonstrated experimentally that AVR-Pita1 from O-137 can result in avirulence in virulent U.S. field isolates. These results



**Fig. 4 – Amino acid sequence alignment of AVR-Pita1 variants. Single-letter amino acid codes are used. Putative metalloprotease motif is indicated and different amino acids are highlighted with different shadings.**

suggest that field isolates in the U.S. carry functional avirulence alleles toward Pi-ta-carrying rice cultivars. Aligning AVR-Pita1 from O-137 with other U.S. AVR-Pita1 variants revealed 92.4% amino acid sequence identity among the predicted AVR-Pita1 proteins (Fig. 4). A total of 11 amino acid differences were identified in AVR-Pita1 alleles of 8 common U.S. races (isolates).

Isolates carrying these AVR-Pita1 variants showed no change in pathogenicity toward Pi-ta-carrying rice cultivars, suggesting that these isolates carry functional AVR-Pita1 variants (Fig. 4).

Previously, it was demonstrated that one amino acid residue of the AVR-Pita1 protease motif determines the degree of avirulence [10,12,13,33]. Additionally, Böhnert et al. [4] found

that a mutation in the putative catalytic site of the B-ketoacyl synthase domain of *ACE1* in the *M. oryzae* avirulence gene *ACE1* abolished the recognition of the fungus by the resistant plant. Tosa et al. [34] determined that selection during the evolutionary process maintained AVR1-Co39's specificity of recognition by cultivar CO39. In the present study, most of the functional portion of the AVR-Pita1 effector was highly conserved, whereas 7.6% represented a polymorphic region including amino acid substitution V173I within the protease motif. However, although V173I lies in the zinc metalloprotease motif, valine and isoleucine are both hydrophobic, resulting in no functional alteration, given that all isolates containing these AVR-Pita1 variants were avirulent to rice germplasm with Pi-ta. This finding suggests that the amino acid variation in U.S. field isolates has no influence on the avirulence activity of AVR-Pita1. We suggest that these polymorphic regions including V173I of the AVR-Pita1 protein are not critical for protease activities.

We demonstrated that AVR-Pita1 from a Chinese isolate can be used to trigger Pi-ta-mediated resistance in virulent U.S. isolates. It is possible that AVR-Pita1 is involved in pathogenicity as a metalloprotease. To determine whether increased copy numbers of AVR-Pita1 changed pathogenicity, transformants with multiple copies of AVR-Pita1 were inoculated on rice cultivars that do not carry Pi-ta. In repeated inoculations, no differences in pathogenicity were observed relative to that of wild-type field isolates. During these studies, two of the avirulent transformants with multiple copies of AVR-Pita1 exhibited a slight reduction of spore production under standard culture conditions, suggesting that these transformants would not survive under natural conditions. However, no changes in pathogenicity of these two transformants on rice cultivars that lack Pi-ta were observed (data not shown). Taking all results together, we suggest that AVR-Pita1 is involved in fitness but is not a critical gene for pathogenicity.

In this study, various gene copy numbers of AVR-Pita1 were identified in most of the transformants. However, the level of avirulence of these transformants remained unchanged when compared with other transformants. In fact, all of the transformants became avirulent, suggesting that the position and arrangement of AVR-Pita1 had no effect on the level of avirulence. Previously, Khang et al. [11] identified three members in the AVR-Pita gene family and confirmed the function of each member as well as their promoters by transferring different combinations of the coding regions and promoter regions. In their study, both AVR-Pita1 and AVR-Pita2 conferred avirulence on isolates virulent toward Pi-ta-containing rice cultivars but AVR-Pita3 failed to do so [11]. These findings are consistent with the predicted role of AVR-Pita1 as an elicitor interacting specifically with the Pi-ta protein in triggering resistance in plant cells [12,13].

Major R gene-mediated resistance can be robust and complete, but may not be long-lasting. That Pi-ta has been defeated by race IE1k suggests an urgent need for exploring novel approaches. In this study, we altered isolates by converting isolates back to their presumed wild-type state. When this was done, the isolates were no longer able to infect Pi-ta-carrying cultivars. For the first time, we experimentally demonstrated that Pi-ta in the U.S. cultivars recognizes the original AVR-Pita identified from a Chinese isolate O-137 and initially named AVR2-YAMO. Our findings also suggest that the development of

a novel race carrying the AVR-Pita1 allele from isolate O-137 of the pathogen could allow the development of rice lines that have more effective, or durable, resistance to the rice blast pathogen.

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